

Quantitative Analysis of Endocrine Disruptors by Comprehensive Two-Dimensional Gas Chromatography

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Comprehensive two-dimensional gas chromatography has been successfully applied to the analysis of various organic contaminants; however, few studies report its use in the analysis of endocrine disrupting compounds. These compounds are suspected to cause dysfunction of the endocrine system in humans and animals. This work describes the development of a method to analyse dibutylphthalate, benzylbutylphthalate, nonylphenol and octylphenol in water using solid-phase microextraction (SPME) and comprehensive two-dimensional gas chromatography with a flame ionisation detector. The merit parameters of the comprehensive two-dimensional gas chromatography with flame ionisation detector method were weighed against a gas chromatography/mass spectrometry-single ion monitoring method of endocrine disrupting compounds analysis. The compounds were evaluated over a concentration range of 0.2 to 6.0 µg L⁻¹. The use of a two-dimensional chromatography method proved to be advantageous in analysing endocrine disrupting compounds, according to the observed increase of the signal relative to the noise and peak resolution.

Keywords: alkylphenols, comprehensive two-dimensional gas chromatography, gas chromatography/mass spectrometry, endocrine disrupting compounds, phthalates

Introduction

Organic micro-contaminants are often found in food, surface water and other environmental matrices. These compounds are usually recalcitrant and some are suspected of causing dysfunction in the endocrine systems of humans and animals. Compounds with such characteristics are classified as endocrine disrupting compounds (EDC).¹⁻³ Studies show that EDC can cause foetal malformation and changes in the growth and development of animals.⁴⁻⁶ In addition to causing endocrine disruption effects, some EDC are suspected to bioaccumulate in various fishes and fresh-water organisms.^{7,8} The European Commission cited several studies and populated a list of 562 compounds that are either suspected or proven endocrine disruptors.⁹ This work focuses on the study of some EDC that are classified as industrial chemicals, including phthalates and

alkylphenols. Some phthalates, such as dibutylphthalate (DBP) and benzylbutylphthalate (BBP), are used as plasticisers in polymers. These compounds can leach into food and water when such plasticisers are used in package formulation.¹⁰ Alkylphenols, such as 4-nonylphenol (4-NP) and 4-octylphenol (4-OP), are degradation products of alkylphenol polyethoxylates, a class of compounds commonly used as nonionic surfactants in domestic and industrial applications.¹¹ Nonylphenol ethoxylate, an alkylphenol ethoxylate widely used in industry, is present in the formulation of various pesticides. The residue of such pesticides can contaminate food because nonylphenols and their main metabolites do not degrade rapidly.^{12,13} The alkylphenols are also used as monomers in the manufacture of phenolic resins, which are widely used in thermoplastic products, including products that come in contact with food.¹⁴ These compounds are of great concern because they can be harmful even at low concentrations. Therefore, methods that enable quantification of trace

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levels of EDC are required. Gas chromatography coupled with mass spectrometry (GC/MS) in full scan and single ion monitoring (SIM) modes have been used to determine EDC concentration in several matrices.¹⁵⁻¹⁷ Comprehensive two-dimensional gas chromatography (GC×GC), which has been applied to the quantitative analysis of various organic compounds, is a viable alternative to GC/MS.¹⁸ Djokic *et al.*¹⁹ showed that GC×GC with a flame ionisation detector (FID) can be successfully applied to the quantification of organic biodiesel compounds. Other studies also report the application of GC×GC to biological matrices. Using GC×GC-FID, Amorim *et al.*²⁰ attained detection limits as low as 0.03 to 0.18 µg L⁻¹ in the analysis of polycyclic aromatic hydrocarbons in urine. Andrews and Paterson²¹ showed that GC×GC/MS can be used to quantify illicit drugs present in low concentrations in blood samples. GC×GC provides an efficient separation, which enables the use of lower-cost universal detectors, such as FID. The use of FID in one-dimensional gas chromatography does not allow structural elucidation of the compounds. In complex samples, there is a high probability that sample analytes and interferents co-elute. Co-elution often impairs the analysis of the compounds even in simple samples such as water, precluding quantitative purposes. The disadvantage in using FID may be offset by the greater separation of compounds obtained by a two-dimensional chromatographic method. The use of two columns with different polarities and the cryo-focusing step increases the separation of the compounds and the resolution of the peaks respectively.²² Moreover, co-elution can be avoided, and matrix effects can be reduced or even eliminated. GC×GC is thus a consolidated technique to analyse complex matrices; nevertheless, its application in analysing endocrine disruptors is still little reported. Studies employing this technique in the analysis of EDCs are mostly qualitative. Studies performed in complex mixtures of alkylphenols mainly show the capacity GC×GC has for distinguishing isomers.^{23,24}

Therefore, the objective of this study was to develop a method to quantify dibutylphthalate, benzylbutylphthalate, 4-nonylphenol and 4-octylphenol in aqueous matrices using GC×GC-FID. The parameters of this method have been evaluated and compared with the results of a GC/MS method operating in SIM mode.

Experimental

Chemicals and reagents

Authentic standards of DBP, BBP, 4-NP and 4-OP were purchased from Sigma Aldrich (St. Louis, MO, USA). Stock solutions were prepared for each compound to a final

concentration of 2000.0 mg L⁻¹ using high-performance liquid chromatography (HPLC)-grade ethanol from J. T. Baker (Xalostoc, Edo MEX, Mexico) and ultra-pure water from purifier Elga (Woodridge, Illinois, USA, Purelab Classic UVMK2 model). The stock solution used in the GC×GC-FID experiments was prepared in HPLC-grade methanol from Merck (Darmstadt, Germany) and ultra-pure water from Diwer Technologies (Lisboa, Portugal, model W1 purifier).

Solid-phase microextraction (SPME) procedure

Cold fibre-solid phase microextraction (CF-SPME)

The experiments were performed using standards of DBP, BBP, 4-NP and 4-OP, which were prepared in ultra-pure water. Compounds were extracted using cooled polyacrylate fibre (85 µm) with liquid nitrogen in an in-house system (CF-SPME).²⁵

The sample (20.0 mL) was placed in a 22 mL vial and subjected to continuous stirring with a magnetic bar, which was placed inside the vial. The compounds were extracted by direct immersion of the cooled fibre in manual mode. The procedure was performed at a vial temperature of 65 °C for 30 min. The samples were analysed by GC/MS.

Conventional SPME

Sample preparation for conventional SPME was performed similarly to the procedure for cold fibre-SPME; however, the fibre cooling is precluded and the final volume of the sample was 15.0 mL. The samples were analysed by GC×GC-FID. The use of conventional SPME will be discussed later.

Gas chromatography analysis

GC/MS

The GC/MS is equipped with a mass analyser-type ion trap (Finnigan Trace GC/PolarisQ, Thermo). The chromatographic analysis was performed using the splitless injection mode for 2 min on an HP-5MS Agilent column (30 m × 0.25 mm × 0.25 µm) with an injector temperature of 250 °C and a helium flow of 1.2 mL min⁻¹. The initial column temperature was 105 °C and was maintained at that temperature for 1 min. The temperature was then ramped to 180 °C at a rate of 3 °C min⁻¹, then maintained at 180 °C for 4 min. The temperature was then increased to 290 °C at a rate of 10 °C min⁻¹ and then maintained at 290 °C for 0.5 min. The total running time was 41.5 min. The acquired data were processed using the software X-Calibur.

The analysis was performed in SIM mode with an electron ionisation energy of 70 eV. For 4-OP, the monitored

ions were at m/z of 107, 108 and 206. For 4-NP, the monitored ions were at m/z of 107, 108 and 220. For DBP, the monitored ions were at m/z of 41, 149 and 150. Lastly, for BBP, the monitored ions were at m/z of 91, 149 and 206.

GC×GC-FID

GC×GC analyses were performed on an Agilent gas chromatograph (model 7890) equipped with a dual stage jet cryogenic modulator (licensed from Zoex), in which nitrogen is used for cooling, and flame ionisation detector, where the hydrogen flow was set at 35 mL min⁻¹ and the air flow at 350 mL min⁻¹. The analyses were performed in splitless mode for 2 min. The injector temperature was set at 250 °C; the carrier gas (H₂) flow rate was 1.5 mL min⁻¹. The first dimension used a Restek Rtx 5 (5% diphenyl/95% dimethyl polysiloxane) (10 m × 0.18 mm × 0.20 µm film thickness d_f) column with a temperature ramp starting at 60 °C for 1 min and increasing to 170 °C at a rate of 10 °C min⁻¹. The temperature was then maintained at 170 °C for 2 min and increased to 280 °C at a rate of 20 °C min⁻¹. The temperature was maintained at 280 °C for 0.5 min. The second dimension used an Agilent DB17 ((50%-phenyl)-methylpolysiloxane) column (1.35 m × 0.10 mm × 0.10 µm d_f) with an initial temperature of 80 °C. The temperature was maintained at 80 °C for 1 min then increased to 185 °C at a rate of 10 °C min⁻¹. The temperature was maintained at 185 °C for 2.5 min then increased to 285 °C at a rate of 20 °C min⁻¹, where it was maintained for 0.5 min. The modulation period was 5 s (duration of the hot pulse was 300 ms), and the modulator temperature was kept at 40 °C above the temperature of the first column. The data were analysed by optimised software Leco Chroma TOF.

Merit parameters

The methods (complete proceeding of extraction and chromatographic analyses) were evaluated in accordance with the EURACHEM parameters.²⁶ We evaluated the precision parameters via intra- and inter-day assays, the linearity, the limit of detection (LOD) and the limit of quantitation (LOQ). The calibration curve was constructed with six different concentrations of each analyte; each concentration was measured in triplicate. The intra- and inter-day assays were evaluated at two concentrations of the calibration curve (1.0 and 5.0 µg L⁻¹). To determine the precision of the method, 10 sample replicates of each concentration were injected and measured over a short time interval (same day) for the intra-day assay. The samples were injected and measured over a long time interval (different days) for the inter-day assay. The limit

of detection was evaluated by injecting 10 replicates of the blank (ultrapure water). The limit of quantitation was determined using the lowest point on the calibration.

Results and Discussion

Extraction method

The compounds of this study have varying polarities; therefore, compared to the other fibres tested, polydimethylsiloxane 100 and 7 µm, carboxen/polydimethylsiloxane 75 µm, polydimethylsiloxane/divinylbenzene 65 µm (results not shown), the polyacrylate fibre presented higher extraction yields and was used in the extraction procedure. The cooled fibre was applied to the GC/MS analyses to increase the extraction efficiency of the compounds. Notably, cooling increases the peak areas obtained for lower concentration levels, making it possible to quantify the compounds at lower concentrations. However, when GC×GC was used for analysis, the high sensitivity already obtained for low concentration levels makes the use of a cooling system unnecessary. Through cooling, the fibre increases the efficiency of analyte extraction from the sample. Nevertheless, the use of liquid nitrogen during sample preparation increases the cost of the analysis, which poses a disadvantage in the application of the CF-SPME for routine analysis. We can observe an increased signal/noise ratio when GC×GC is used. When GC/MS was used the signal/noise ratio was 695, 1261, 156 and 127 for OP, NP, DBP and BBP, respectively, for concentration of 5.0 µg L⁻¹. On the other hand, using the same concentration GC×GC signal/noise ratio values were 9589, 11875, 3976 and 1771 for OP, NP, DBP and BBP, respectively. The increase in resolution, which will enable the detection and analysis of trace level compounds, is the result of the presence of two columns with different polarities. Additionally, the matrix effect is clearly reduced due to the separation obtained. Co-elutions were avoided and good shaped spots are obtained.

Method performance

The GC×GC-FID analysis was performed in a range from 0.2 to 6.0 µg L⁻¹ for DBP and from 0.5 to 6.0 µg L⁻¹ for BBP, 4-NP and 4-OP. Higher concentrations were not evaluated. The colour plot obtained for standards of DBP, BBP, 4-NP and 4-OP (5.0 µg L⁻¹) is shown in Figure 1.

When developing the analysis method for endocrine disruptors HPLC-grade solvents and ultra-pure water were used to avoid any contamination. The presence of contaminants was also minimised due to the sample

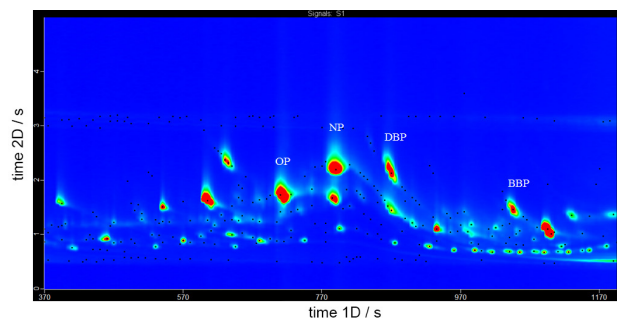


Figure 1. Colour plot obtained by GCxGC-FID of endocrine disruptors extracted by SPME; see conditions in the text.

preparation procedure used by means of SPME. However as shown in Figure 1 after a GCxGC-FID run, even the use of high purity solvents was not enough to completely eliminate many contaminants. This can be observed through the presence of several other peaks of lower intensity in the colour plot. Although the four studied EDC present co-elution with interferents in the first dimension column, all four compounds are well separated from these contaminants in the second dimension column. A clear separation between target compounds and contaminants is achieved in a 20 min run. This efficiency in separation reducing the matrix effect allows the use of GCxGC-FID for trace analysis and quantitation in complex samples, precluding the use of mass spectrometry. In fact, using mass spectrometry in SIM mode one monitored only the ions of interest, losing the capability to verify the effectiveness of the isolation/extraction procedure during the sample preparation step. GC/MS/SIM is generally blind to matrix effects by the absence of any other compounds except those chosen by mass selective monitoring. In a long-term routine method this is important, since the MS system is thus prone to lose sensitivity associated with MS source contamination. Using GCxGC-FID one can immediately verify and correct any loss of sensitivity and eventually diagnose its origin. Also the GCxGC-FID system maintenance is easier compared with any MS system and also less expensive. Moreover, since GC/MS, in order to provide sufficient separation, needs

to be performed in a 30 m long column, compared with a 11.35 m long column set (1st and 2nd dimension columns together) for the GCxGC-FID experiment, longer analysis time are obtained, 41.5 min compared with the 20 min necessary for the GCxGC-FID run.

The premises of linear regression were assessed by applying the model to the ordinary least squares. The linearity of the curves was evaluated using Ryan-Joiner statistical tests to evaluate the normality of the residuals and the Durbin-Watson statistic to evaluate the independence of the residuals. The Brown-Forsythe test was used to evaluate the homogeneity of the residuals, and the analysis of variance (ANOVA) test was used to evaluate the significance regression and deviation from linearity.²⁷ An interval of 95% confidence was admitted. The tests were applied using the software Excel. The analytical curves obtained using GCxGC-FID showed that the residues were homoscedastic and independent with normal distribution for all compounds. The data showed that the regression was significant. The deviation from linearity was not observed for the curves obtained. Therefore, the analytical curves were constructed based on the linear regression model. The equation of the curve was obtained from area of each standard analysed. The equations of the curves, the coefficients of determination and the respective standard deviations of intercepts and slopes are shown in Table 1.

The GC/MS analysis was performed over a range of concentrations from 0.2 to 6.0 $\mu\text{g L}^{-1}$ for DBP and 4-NP, from 0.5 to 6.0 $\mu\text{g L}^{-1}$ for BBP and from 0.2 to 8.0 $\mu\text{g L}^{-1}$ for 4-OP (Table 1). These values were determined by evaluating the linear range for all compounds; all compounds were tested over a range of concentrations from 0.2-8.0 $\mu\text{g L}^{-1}$. The statistical analysis of the results showed that the residues obtained in the construction of the curves of DBP, BBP and 4-NP are homoscedastic, independent and follow a normal distribution in an interval of 95% confidence. The ANOVA test showed that the regression is significant and that there is no deviation from linearity. Therefore, a linear regression can be applied. For 4-OP, the residues were heteroscedastic and independent with

Table 1. Linear regression equation, Pearson coefficient of determination and standard deviations of intercepts and slopes for the EDC studied by GC/MS and GCxGC-FID

Compound	GC/MS				GCxGC-FID			
	Equation	R ²	SD intercept	SD slope	Equation	R ²	SD intercept	SD slope
DBP	$y = 5.5 \times 10^4 x + 14941^a$	0.9905	5007	1354	$y = 4.0 \times 10^6 x + 86373^a$	0.9894	399034	111446
BBP	$y = 2.9 \times 10^4 x - 4883^a$	0.9854	3322	897	$y = 3.4 \times 10^6 x - 207092^a$	0.9892	338533	91887
4-OP	$y = 3.9 \times 10^5 x - 45043^a$	0.9863	27032	20769	$y = 1.1 \times 10^7 x + 81382^a$	0.9895	1089409	301636
4-NP	$y = 9.2 \times 10^5 x - 368797^a$	0.9886	91188	24629	$y = 9.3 \times 10^6 x - 12089670^a$	0.9893	908534	270073

^ax is given in $\mu\text{g L}^{-1}$.

Table 2. Precision intra- and inter-day assays and LOD and LOQ obtained in the area analysis of EDC (n = 10) studied by GC/MS and GC×GC-FID

Compound	GC/MS						GC×GC-FID					
	1.0 µg L ⁻¹		5.0 µg L ⁻¹		LOD / (µg L ⁻¹)	LOQ / (µg L ⁻¹) $\bar{x} \pm s$	1.0 µg L ⁻¹		5.0 µg L ⁻¹		LOD / (µg L ⁻¹)	LOQ / (µg L ⁻¹) $\bar{x} \pm s$
	Intra / %	Inter / %	Intra / %	Inter / %			Intra / %	Inter / %	Intra / %	Inter / %		
DBP	11.7	11.9	7.6	8.2	0.08	0.12 ± 0.04	16.0	11.7	12.5	15.2	0.07	0.17 ± 0.03
BBP	16.2	22.3	11.0	13.9	0.31	0.68 ± 0.07	26.0	28.4	15.7	18.4	0.26	0.47 ± 0.07
4-OP	15.2	18.8	16.4	19.3	0.11	0.25 ± 0.03	12.9	18.9	11.6	13.0	0.14	0.46 ± 0.06
4-NP	18.7	18.3	15.7	19.2	0.40	0.66 ± 0.03	18.5	17.4	16.3	15.8	0.32	0.55 ± 0.03

normal distribution. Therefore, weighted linear regression was used to construct the calibration curve.

The precision of the developed methods was evaluated by analysing the variation coefficients obtained from 10 replicate injection standards (Table 2). The relative standard deviation (RSD) values by GC×GC-FID obtained for EDC were lower than 20% for all compounds except for BBP at a concentration of 1.0 µg L⁻¹. Probably BBP has a larger RSD due to a lower sensitivity of the technique in the analysis of this compound, that has a greater variation in area obtained in low concentrations. A maximum RSD of 20% is acceptable in the analysis of trace compounds in food and environmental matrices.^{26,28}

The RSD values observed by GC/MS were lower than 20% for all compounds except for BBP, where a 22.3% RSD was observed at a concentration of 1.0 µg L⁻¹ in the inter assay. This higher RSD value can be explained by the lower sensitivity of BBP compared with the other compounds. The deviations obtained at a concentration of 1.0 µg L⁻¹ were generally higher than deviations obtained at a concentration of 5.0 µg L⁻¹. This result is expected because at low concentrations, the areas are smaller, and small deviations have a greater influence on the RSD.

The LOD and LOQ were obtained by analysing sample blank replicates. Phthalates are contaminants that are found in ultrapure water at very low concentrations due to the presence of plastic parts in the water purification system. The concentration of phthalates in water is lower than the lowest measured concentration of the calibration curve and does not interfere in the analysis of samples because it is constant. The LOD and LOQ were obtained according the EURACHEM recommendation. The expressions for both are as follows:

LOD area: mean blank response + 3 standard deviations;

LOQ: the lowest point on the calibration curve.

The LOD and LOQ observed when GC×GC-FID is used were similar to those obtained when GC/MS was employed (Table 2).

The LOD and LOQ obtained for the studied compounds

show that analysis by GC×GC yielded results that were similar to those observed in GC/MS-SIM analysis. GC×GC-FID analysis forwent the cooling of the SPME fibre; however, notably, cooling would likely increase the sensitivity of GC×GC-FID by increasing the signal/noise ratio. The cooling of fiber of the GC×GC-FID would further increase the signal/noise ratio, however, it would also have an increased amount of contaminants extracted, which is not desirable.

Conclusions

Analysing endocrine disruptors by GC×GC in an aqueous matrix yielded similar results to the GC/MS method, thereby validating GC×GC as a routine method to detect and quantify these compounds at low concentrations. The results showed that the GC×GC-FID method has linearity, precision and limit of detection and quantification comparable to GC/MS. GC×GC-FID is an alternative to GC/MS when routine screening and quantitation is aimed. Clearly, GC/MS is always needed for qualitative compound characterisation, but according to our study, no longer mandatory for routine quantification. The sensitivity and resolution of both techniques are equivalent for the analysis of the endocrine disruptors studied. Nevertheless, the GC×GC-FID showed higher separation efficiency and peak resolution. Additionally, GC×GC-FID can operate applying the modulation process only during the retention time periods of the compounds of interest, reducing the operational costs and making the method even more sustainable. Although during this study one chose to modulate all chromatographic run in order to obtain the total chromatographic profile of each sample, in future applications selective modulation will be performed. Another advantage of the GC×GC-FID compared with GC/MS is related to the total time of analysis. In a shorter period of time well separated peaks are obtained. In this study, EDC samples were reconstituted in water, a relatively simple matrix; however, analysis of endocrine disruptors

may be applied to complex matrices as well. The method developed is now being applied to screen food samples.

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